Cloning Via Genome Integration (pCFB255) [1]

Introduction

After codon optimization we planned to put the Subtilisin E gene inside an integrative vector pCFB255[1] for better expression in *Saccharomyces cerevisiae.*

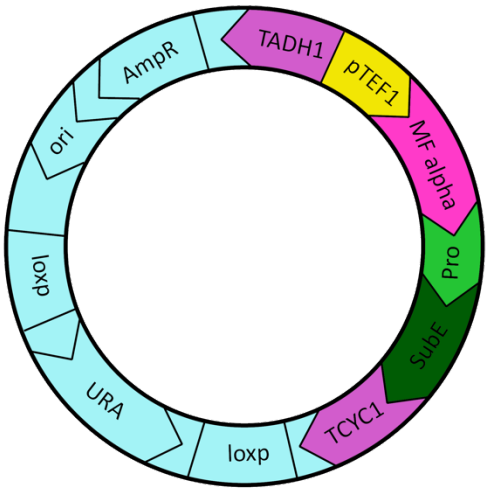

Figure 1: pCFB255[1] with pTEF1 Promoter, secretion tag MFalpha and codon optimized Subtilisin E.

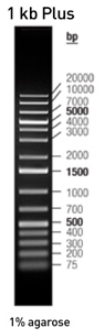

Figure 2: 1kb Thermo scientific ladder

For all agarose gels we used 1% agarose and 1 kb Thermo scientific ladder 08.

Day 1

Primer design for Gibson Assembly

We used the NEBuilder assembly tool to design the suitable primers for the integration vector and optimized Subtilisin E gene for the Gibson Assembly.
The sequence of the codon optimized construct in the parts collection: BBa_K2020023.

Day 2

PCR amplification of Genome integration vector

We amplified the Integration vector pCFB255[1] using primers YP0024 and YP0026 with this following reaction settings according to the PCR protocol:

REAGENTS	VOLUME
Water	14
Polymerase Buffer	2
Forward Primer 10 uM (Y0024)	1
Reverse Primer 10 uM (Y0025)	1
dNTPs	0.5
Template (pCFB255) 53.7 ng/µl	1
Pfu polymerase	0.5
Total Volume	**20**

And the following PCR setting:

ID	98°C	30 sec	
D	98°C	10 sec	X32
A	64-75°C	15 sec	
E	72°C	4 min 30 sec	
FE	72°C	7 min 30 sec	
S	4°C	storage	

Agarose Gel

We ran an agarose gel to see if the PCR product is present and has the right size of about 6000 bp.

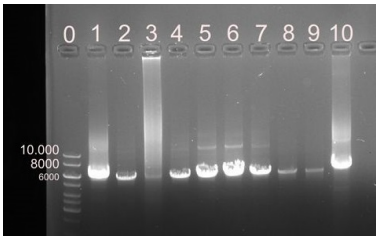

Figure 3: Agarose gel with PCR products from gradient PCR;; 1: 64°C; 2: 64.7°C; 3: 65,7°C; 4: 67°C; 5: 68,4°C; 6: 69,9°C; 7: 71,4°C; 8: 72,8°C; 9: 73,9°C; 10: 74,6°C; 0: Ladder

The bands were at the expected size.

PCR clean up

We performed a PCR clean up of the amplified integration vector pCFB255.

Nanodrop

We did a nanodrop of the cleaned up samples of integration vector pCFB255.
Concentrations were:

1. 131. ng/µl
2. 101.9 ng/µl
3. 116 ng/µl

Day 3

PCR amplification of Gibson Insert

We amplified the codon optimized Subtilisin E with YP0026 and YP0027 with the following reaction settings according to the PCR protocol:

REAGENTS	VOLUME (µl)
Water	14
Polymerase Buffer	2
Forward Primer 10 uM (Y0026)	1
Reverse Primer 10 uM (Y0027)	1
dNTPs	0.5
Template (Subtilisin E) 80.1 ng/µl	1
Pfu polymerase	0.5
Total Volume	**20**

ID	98°C	30 sec	
D	98°C	10 sec	X32
A	58-65°C	15 sec	
E	72°C	1 min 30 sec	
FE	72°C	7 min 30 sec	
S	4°C	storage	

Agarose gel

We prepared an agarose gel to see if the PCR product is present and has the right size of about 1100 bp.

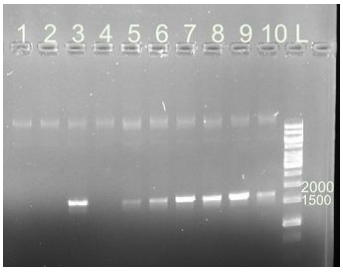

Figure 4: Agarose gel with PCR products from gradient PCR; 1: 58,1°C; 2: 58,5°C, 3: 59,1°C; 4: 59,9°C; 5: 60,8°C; 6: 61,7°C; 7: 62,6°C; 8: 63,4°C; 9: 64,1°C; 10: 64,6°C; L: Ladder

The bands were at the expected size.

PCR clean up

We performed a PCR clean up of the amplified codon optimized Subtilisin E for Gibson assembly.

Nanodrop

We did a nanodrop of the cleaned up samples of codon optimized Subtilisin E for Gibson assembly.

1. 262.6 ng/µl
2. 279.5 ng/µl
3. 151.1 ng/µl

Gibson Cloning

We did Gibson assembly cloning by pipetting the following reaction:

REAGENTS	VOLUME (µl)
Integration Vector (pCFB255) (130ng)	1
Gibson insert(Codon optimized Subtilisin E) (110ng/µl)	2
Water	7
Gibson Assembly master mix	10
Total Volume	**20**

And we kept it at 50°c for 15 minutes incubation and we did the transformation of this Gibson assembly product in *E. coli* according to the protocol.

Day 4

Transformation results

The transformation was successful and the colonies were picked and grown in LB + Amp medium overnight.

Day 5

Plasmid Isolation

We isolated the plasmids from the overnights and sent them for sequencing using the primers YP0019 and YP0030.

Day 6

Sequencing results

The sequencing results of clone 2 were perfect.

Transformation in *S. cerevisiae*

We transformed the clone 2 of pCFB255 with codon optimized Subtilisin E insert into *S. cerevisiae* CENPK2-1D cells and plated on SD-U agar plates.

Day 7

S. cerevisiae overnights:

The transformation of yeast was successful and the colonies were picked for overnights in SD-U medium.

Results

After day 7 we were able to start the test expression tests.

Reference:

[1] Jensen et al. (2013). “EasyClone: method for iterative chromosomal integration of multiple genes in Saccharomyces cerevisiae” FEMS Yeast Research 14(2): 238-248. Provided by Irina Borodina, Technical University of Denmark